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(54) Title: PLANT PROMOTER

(57) Abstract

A promoter which is capable of directing protein expression in roots in plants and which has: (a) the sequence from nucleotide -1616 to nucleotide -1 shown in Figure 1 or a part of the said sequence or (b) a said sequence (a) modified by one or more nucleotide substitutions, insertions and/or deletions and/or by an extension at either or each end.

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# PLANT PROMOTER

The present invention relates to a plant promoter.

Extensin is the best characterised structural protein in the walls of plant cells (Cassab and Varner, Ann. Rev. Plant Physiol. 39, 321-353, 1988). It is a member of a class of highly basic, hydroxyproline-rich glycoproteins present in a wide variety of plants. Extensin has been reported to be present at greatest abundance in sclerenchyma tissues and a variety of functional roles have been ascribed to the protein, including mechanical strengthening and organisation of the cell wall. Extensins vary in their primary structure in different plants, organs and tissues. In some cases, there are two different extensins within the same tissue or cell type, which may be function-related.

As part of a programme to isolate plant genes expressed in an organ-specific manner, mRNA species present in the roots of oilseed rape (Brassica napus L.) were investigated, with the aim of isolating sequences abundant in root but not in other tissues. A family of cross-hybridising sequences present at highly enhanced levels in root has been isolated, characterised, and shown to encode proteins homologous to extensin. This has enabled us to identify a promoter capable of directing the expression of proteins in roots.

Accordingly, the present invention provides a promoter which is capable of directing protein expression in roots of plants and which has:

(a) the sequence from nucleotide -1616 to nucleotide -1 shown in Figure 1 or a part of the said sequence or

(b) a said sequence (a) modified by one or more nucleotide substitutions, insertions and/or deletions and/or by an extension at either or each end.

The invention also provides a DNA fragment comprising such a promoter operably linked to a gene,

typically a heterologous gene encoding a protein. Additionally provided is a vector which comprises a gene encoding a protein under the control of a promoter as above such that the gene is capable of being expressed in a plant cell transformed with the vector. A suitable vector is one in which the promoter is fused directly to the 5'-end of the gene. The vector may further contain a region which enables the gene and the promoter to be transferred to and stably integrated in a plant cell genome. The vector is generally a plasmid.

Plant cells can be transformed with such a vector. The invention therefore further provides plant cells which harbour a promoter as above operably linked to a gene encoding a protein. Transgenic plants may be regenerated from such plant cells. A transgenic plant can be obtained which harbours in its cells a promoter as above operably linked to a gene encoding a protein. Seed may be obtained from the transgenic plants. Plants may in turn be grown from this seed.

The invention additionally provides a method of producing a transgenic plant capable of producing a desired protein in the roots of the plant, which method comprises:

(i) transforming a plant cell with a vector according to the invention, the protein encoded by the gene under the control of the said promoter being the desired protein; and

(ii) regenerating plants from the transformed cells.

In the accompanying drawings:

Figure 1 shows the nucleotide sequence of gene <u>extA</u>, with the predicted amino acid sequence of the rape extensin polypeptide, and the sequence of the promoter of the invention from nucleotide -1616 to nucleotide -1. The complete sequence is the sequence of the 2.7kb HincII-HincII fragment shown in Figure 2. The predicted site of cleavage of the leader sequence is indicated by a colon (:). The

transcription start point is indicated by a circumflex (T^S), and a sequence similar to the consensus "TATA" control box is also indicated. Other sequence features are as indicated on the Figure.

Figure 2 is a restriction map of the oilseed rape genomic clone lambdaB31 (12.7 kb) encoding gene <u>extA</u>, a restriction map of pRlambdaS4, a HindIII-AvaI fragment from lambdaB31 in pUC18, and a sequencing map of the 2.7kb HincII-HincII fragment containing <u>extA</u>. ra and la, indicate right arm and left arm of the EMBL 3 lambda vector, respectively; indicates coding sequence. Sa/H2-A1/Sm/X1 fragment is a part of EMBL 3. Key to restriction site symbols on sequencing map: Sa, Sal I; H3, Hind III; Sm, SmaI; H2, Hinc II; P1, Pst I; AI, AvaI; XI, Xma I; Ns, Nsi I; RI, Rsa I; Ha, Hae III; Ss, Ssp I; Nd, Nde I; Pv, Pvu II.

Figure 3 shows a detailed restriction map of pRlambdaS4, a sub-clone from λB31 comprising a HindIII-AvaI fragment of lambdaB31 in pUC18. shows the location and direction of transcription of the extensin coding sequence. There are no sites in the insert for PvuI, XhoI, BscI, BamHI, BglII and EcoRV.

Figure 4 shows a simplified restriction map of the sub-clone pRlambdaS4; indicates the position of the extensin coding sequence and indicates the region containing the promoter conferring root expression.

Figure 5 is a map of the hybrid gene employed in Example 2; indicates the nopaline synthase (NOS) terminator.

Figure 6 is a map of the hybrid gene from Figure 5 in the vector BIN 19.

Figure 7 shows the rape extensin promoter on a HaeIII fragment excised from lambdaB31, as described in Example 3.

Figure 8 shows the fusion construct comprising the rape extensin promoter and the glucuronidase gene coding

sequence, prepared in Example 3.

The full length promoter of the invention is composed of the sequence upstream of the oilseed rape extA gene, from nucleotide -1616 to nucleotide -1 in Figure 1, base 1 being the adenine (A) base of the CAT transcriptional start codon for the extA gene. The promoter may be obtained from a clone, lambdaB31 by excision on a 4.5kb HindIII-AvaI fragment (Figure 3). Clone lambdaB31 was obtained from a genomic library prepared from oilseed rape (Brassica napus L). The HindIII-AvaI fragment was subcloned in pUC18 as pRlambdaS4 from which the promoter can be excised as a 0.96kb HaeIII fragment. E. coli DH5α harbouring pRlambdaS4 and bacteriophase lambdaB31 have been deposited at the National Collection of Industrial and Marine Bacteria, Aberdeen, GB on 8 March 1990 under accession numbers NCIMB 40265 and NCIMB 40266.

A part of the full length sequence from nucleotide -1616 to nucleotide -1 and modified forms of the full length or part sequence are alternative promoters according to the invention. The full length or part promoter sequence may be modified by one or more nucleotide substitutions, insertions and/or deletions and/or by an extension at either or both ends. Any part or modified promoter sequence must however still be capable of acting as a promoter in the roots of plants. When the full length sequence or a part of the full length sequence, i.e. an unmodified sequence, is modified typically there is a degree of homology of at least 60% between a modified sequence and the unmodified natural sequence. The degree of homology may be at least 75%, at least 85% or at least 95%.

A part of the full length promoter sequence may be obtained by use of restriction endonucleases and/or exonucleases. The promoter may be obtained as a 0.96 kb fragment by treating the sequence shown in Figure 1 with HaeIII. A modified sequence may be obtained by introducing changes into an unmodified promoter sequence. This may be

achieved by any appropriate technique, including restriction of the natural sequence with an endonuclease, insertion of oligonucleotide linker adapters, use of an exonuclease and/or a polymerase and site-directed mutagenesis.

Whether a part of the full length promoter sequence or a modified sequence is capable of acting as a promoter may be readily ascertained by experiment. The putative promoter sequence is fused to the glucuronidase coding sequence in the promoter-less binary vector pBI101 at the SmaI site as described in Example 3. Following the procedure of Example 3, expression of glucuronidase is investigated in the transgenic hairy roots produced.

The promoter may be operably linked to a gene, typically a heterologous gene, encoding a protein. The heterologous gene may encode any protein it is desired to express. "Heterologous" means that the gene is not naturally operably linked to the promoter, i.e. a heterologous gene is not the oilseed rape extA gene. The protein may additionally comprise a transit peptide sequence at its N-terminus, encoded within the "heterologous" gene sequence.

The promoter is typically used to express proteins in the roots of plants. The protein whose expression is controlled by the promoter may be for example a protein conferring biological control of pests or pathogens, in particular pests or pathogens to which the roots of plants are susceptible.

The promoter sequence may be fused directly to a gene or via a linker. The linker sequence may comprise an intron. Excluding the length of any intron sequence, the linker may be composed of up to 45 bases, for example up to 30 or up to 15 bases.

DNA fragments and vectors can be prepared in which the promoter is operably linked to a gene, typically a heterologous gene. The fragments and vectors may be single or double stranded. Plant cells can be transformed by such

fragment by direct DNA uptake, or by way of such a vector. The vector incorporates the gene under the control of the promoter. The vector contains regulatory elements capable of enabling the gene to be expressed in a plant cell transformed with the vector. Such regulatory elements include, besides the promoter, translational initiation and/or termination sequences. The vector typically also contains a region which enables the gene and associated regulatory control elements to be transferred to and be stably integrated in the plant cell genome.

The vector is therefore typically provided with transcriptional regulatory sequences and/or, if not present at the 3'-end of the coding sequence of the gene, a stop codon. A DNA fragment may therefore also incorporate a terminator sequence and other sequences which are capable of enabling the gene to be expressed in plant cells. An enhancer or other element able to increase or decrease levels of expression obtained in particular parts of a plant or under certain conditions may be provided in the DNA fragment and/or vector. The vector is also typically provided with an antibiotic resistance gene which confers resistance on transformed plant cells, allowing transformed cells, tissues and plants to be selected by growth on appropriate media containing the antibiotic.

Transformed cells are selected by growth in an appropriate medium. Plant tissue can therefore be obtained comprising a plant cell which harbours the gene under the control of the promoter , for example in the plant cell genome. The gene is therefore expressible in the plant cell. Plants can then be regenerated which include the gene and the promoter in their cells, for example integrated in the plant cell genome, such that the gene can be expressed. The regenerated plants can be reproduced and, for example, seed obtained. Root-specific expression of proteins can therefore be directed by the present promoter in plants. Alternatively, transformed roots may be grown in culture for

the purposes of production of proteins, especially plant proteins.

A preferred way of transforming a plant cell is to use Agrobacterium tumefaciens containing a vector comprising the promoter operably linked to the gene encoding a protein it is wished to express. A hybrid plasmid vector may therefore be employed which comprises:

(a) the gene under the control of the promoter and other regulatory elements capable of enabling the gene to be expressed when integrated in the genome of a plant cell;

(b) at least one DNA sequence which delineates the DNA to be integrated into the plant genome; and

(c) a DNA sequence which enables this DNA to be transferred to the plant genome.

Typically the DNA to be integrated into the plant cell genome is delineated by the T-DNA border sequences of a Ti-plasmid. If only one border sequence is present, it is preferably the right border sequence. The DNA sequence which enables the DNA to be transferred to the plant cell genome is generally the virulence (vir) region of a Ti-plasmid.

The gene and its transcriptional and translational control elements, including the promoter, can therefore be provided between the T-DNA borders of a Ti-plasmid. The plasmid may be a disarmed Ti-plasmid from which the genes for tumorigenicity have been deleted. The gene and its transcriptional and control elements, including the promoter, can, however, be provided between T-DNA borders in a binary vector in trans with a Ti-plasmid with a vir region. Such a binary vector therefore comprises:

(a) the gene under the control of the promoter and other regulatory elements capable of enabling the gene to be expressed when integrated in the genome of a plant cell; and

(b) at least one DNA sequence which delineates the DNA to be integrated into the plant genome.

Agrobacterium tumefaciens, therefore, containing a

hybrid plasmid vector or a binary vector *in trans* with a Ti-plasmid possessing a *vir* region can be used to transform plant cells. Tissue explants such as stems or leaf discs may be inoculated with the bacterium. Alternatively, the bacterium may be co-cultured with regenerating plant protoplasts. Plant protoplasts may also be transformed by direct introduction of DNA fragments which encode the gene of interest and in which the promoter and appropriate other transcriptional and translational control elements are present or of a vector incorporating such a fragment. Direct introduction may be achieved using electroporation, polyethylene glycol, microinjection or particle bombardment.

Plant cells from monocotyledonous or dicotyledonous plants can be transformed according to the present invention. Monocotyledonous species include barley, wheat, maize and rice. Dicotyledonous species include tobacco, tomato, sunflower, petunia, cotton, sugarbeet, potato, lettuce, melon, soybean, canola (rapeseed) and poplars. Tissue cultures of transformed plant cells are propagated to regenerate differentiated transformed whole plants. The transformed plant cells may be cultured on a suitable medium, preferably a selectable growth medium. Plants may be regenerated from the resulting callus. Transgenic plants are thereby obtained whose cells harbour the promoter operably linked to the gene encoding the protein it is wished to express, for example integrated in their genome. The gene is consequently expressible in the cells. Seed from the regenerated plants can be collected for future use, and plants grown from this seed.

The following Examples illustrate the invention.

EXAMPLE 1

1. A family of cross-hybridising cDNA clones was isolated from a cDNA library produced with poly(A)+RNA from the roots of oilseed rape (*Brassica napus* L.). The clones were selected as abundantly expressed in root by differential screening of the root cDNA library with cDNA

probes prepared from root, green leaf, etiolated leaf and developing seed. mRNA species corresponding to the selected abundant clones were expressed in roots at levels of at least 400 x those in other organs, as shown by Northern blot analysis and RNase protection assays. One of the cDNA clones was designated $pRR_t566$.

2. An extensin gene designated extA, was obtained from an oilseed rape (Brassica napus L.) genomic library screened with $pRR_t566$ at high stringency. The gene is a member of a multigene family, consisting of about three members per haploid genome with strong homology to the probe, and a further twenty or so members of weaker homology. The isolated gene, although not identical to the cDNA probe, was also found to be specifically expressed in roots, and was transcribed into a mRNA species of approximately 1300 nucleotides in size. A single transcription start was identified by S1 mapping. A complete nucleotide sequence of the extA gene and its flanking regions was determined. This enabled the gene promoter to be identified. In more detail:

Identification of extensin genes in genomic DNA

The cDNA species from rape root, $pRR_t566$, previously identified as encoding a protein homologous to extensin, was used as a probe on Southern blots of restrictions of rape genomic DNA. The insert of $pRR_t566$ hybridised to a large number of genomic fragments in all restriction digests, but the strength of hybridisation to all fragments was not equal; 1-2 fragments in all digests hybridised at an intensity at least five times that of the other bands. Copy number reconstructions indicated that the strong bands corresponded to approx. 2-3 gene copies per haploid genome; the patterns can be consistently interpreted to give a total of 3 genes per genome highly homologous to this probe. The weakly hybridising fragments were present at less than one estimated copy per haploid genome, and must therefore represent genes that are only partly homologous to the extensin probe. The total sequences detected by this

probe make up a large multigene family, of which three genes, determined to be closely homologous to $pRR_t566$, form a sub-family. In confirmation of these conclusions, when a 3' flanking sequence probe (240 bp) was prepared from $pRR_t566$, and hybridised to genomic blots as above, hybridisation of this probe to the strongly hybridising bands was identical to that using the whole cDNA insert as a probe, except hybridisation to the $pRR_t566$ weakly hybridising bands was largely eliminated.

<u>Isolation and characterisation of a genomic clone containing an extensin gene</u>

An oilseed rape genomic library, constructed by inserting random Sau 3A fragments of genomic DNA, into lambda EMBL3, was screened at low stringency with a probe prepared from the insert of $pRR_t566$. This cDNA clone was isolated from a library prepared from rape root mRNA, and encoded part of an extensin polypeptide. Forty positive phage plaques were identified, and were extensively plaque-purified. The isolated clones were then re-screened under high stringency conditions (0.1 x SSC, 0.1% SDS at 65°C), when only one phage clone gave continued strong hybridisation to the cDNA probe. This clone, lambdaB31, was selected for further study.

DNA from the selected phage was isolated and characterised by restriction and Southern blotting, using $pRR_t566$ as a probe. The restriction map of lambdaB31 obtained is shown in Fig. 2, and shows that the clone contains an insert of 12.7 kb. The region of DNA hybridising to the cDNA probe was localised to a fragment of 1.0 kb (Nsi I-Pst I) adjacent to the left arm of the vector. To further characterise the gene present on this clone, restriction with Dde I and Rsa I was carried out, followed by Southern blotting and probing with the cDNA as before. These two enzymes were chosen because they restrict the homologous cDNA sequences at multiple sites, corresponding

to the repeats in the coding sequence, and also generate unique diagnostic fragments from the 3' non-coding region of the sequence. Although the genomic clone contained the 84 bp restriction fragments generated from the region of $pRR_t566$ encoding the 28 amino acid repeat sequences, the diagnostic fragments specific to $pRR_t566$ were not present, suggesting that the gene present was not identical to the cDNA. DNAs from a further ten phage clones were purified and similarly screened; however, none of the clones contained diagnostic fragments identical to $pRR_t566$ and were not considered further in the present study.

To further characterise the gene present on lambdaB31, a Southern blot of insert DNA was hybridised with a probe prepared from a second rape root extensin cDNA clone, $pRR_t592$; this probe contained only 39 bp of sequence identified as encoding a putative leader sequence of an extensin precursor polypeptide and hybridised to the same 1.0 kb fragment as was detected by the $pRR_t566$ probe. A 3' flanking sequence probe from $pRR_t566$ was prepared by subcloning a Hinc II-EcoRI fragment into pUC 18. This probe contained no coding sequence, which hybridises very strongly due to its G-C rich nucleotide composition. The 3' probe failed to hybridise to lambdaB31 DNA suggesting that this clone contained the 5' end and coding sequence of an extensin gene, but either lacked the 3' flanking sequence, or was divergent in the 3' flanking region.

When the 1.0 kb fragment of lambdaB31 was labelled and used as a probe on Southern blots of restricted rape genomic DNA, similar results (not shown) to those obtained when $pRR_t566$ was used as a probe, were obtained. A 1.85 kb Hinc II fragment of genomic DNA which was present at 1 copy per haploid genome, corresponded to the expected Hinc II fragment predicted from the lambdaB31 restriction map.

Sequence of extensin gene extA

Approximately 2.7 kb of DNA sequence of the genomic

clone lambdaB31 was fully determined. The 5.3kb HindIII-AvaI fragment from lambdaB31 was subcloned into pUC18 (Yanisch-Perron et al, Gene 33, 103-119, 1985) to give pRlambdaS4 (Figures 2 and 3) from which the 2.7kb HincII-HincII fragment was sequenced (Figure 2). The sequencing map for this region is shown in Fig. 2, and the complete determined sequence is given in Fig. 1. The sequence contains an open reading frame predicting a polypeptide of 299 residues. The first 23 residues of the sequence encode a leader sequence, when compared with other extensin sequences and as defined by the rules of von Heijne (1985, J. Mol. Biol. 184, 99-105). The remainder of the coding sequence encodes a highly proline-rich polypeptide of 276 amino acids.

The promoter of the invention is the 5' flanking sequence of the extA gene as shown in Figure 1. This sequence contains a sequence (CTATATAAA) closely homologous to the consensus "TATA" box for plants seventy-four bases 5' to the initiation codon. Apart from this no significant features can be recognised. Comparison of this 5' flanking sequence with that of the carrot extensin gene pDC5A1 (Chen and Varner, EMBO J. 4, 2145-2151, 1985) reveals no strongly conserved sequence regions, apart from the "TATA" box.

Expression of extA

To confirm that extA represents an expressed gene, and to determine its transcription start, a series of S1 mapping experiments were carried out. Fragments of extA 5' end-labelled at a Dde I site (base 71) and extending in a 5' direction to the Nsi I site (base -74) or the Dde I site (base -237) were isolated and hybridised to poly(A)+RNA from rape roots. After treatment with S1 nuclease, the protected fragments were sized by polyacrylamide gel electrophoresis. In both cases, protected fragments of 66-75 bases were obtained with the strongest band corresponding to the underlined base in the sequence TAAGAGCATCAAAC, which was

designated base +1 (indiated in Figure 1 by T^S). This sequence is in good agreement with the consensus transcription start in plants, -CATC- and is at a congruous distance from the "TATA" box (34 bases). No other protected fragments that were not also present in the controls were observed. A further probe, labelled at the Nde I site at base -86 and extending to the Nde I site at base -453, was prepared and hybridises as above. No protected fragments of this probe were observed, although a small amount of intact probe was present in both the experimental and control hybridisations. These results suggest that the determined transcription start is the only start site in extA, in contrast to the carrot extensin gene where two transcription start points were observed.

To confirm the above conclusions, and to investigate the organ-specificity of expression of extA, fragments of the gene were used as probes on Northern blots of RNA from different rape organs. A probe consisting of the complete coding sequence of the gene, and extending to base -74, was hybridised to RNA from four rape organs: root, green leaf, etiolated leaf and developing seed. This probe hybridised to two mRNA species in root, of approx. 1300 and 1480 bases, and hybridised only very weakly to a mRNA species in developing seeds, of approx. 1600 bases. This hybridisation pattern is very similar to that given by the cDNA species $pRR_t556$ (and closely homologous species), and rehybridisation of the same blot to the cDNA probe confirmed that the same bands were detected by both probes. If the size of 3' flanking sequence of $pRR_t566$ is added to that of the sequence of extA, the completed gene would be predicted to produce an mRNA species of approx. 1250 bases, assuming a poly(A) tail of 30 bases is added to the cDNA sequence. The observed mRNA species of approx. 1260 bases in rape roots can therefore be suggested to be the product of extA. A very low level of hybridisation of the extA probe to a large mRNA species (approx. 4.6 kb) present in root was also

observed.

EXAMPLE 2: Expression of the extA in Transgenic Tobacco

The original rape extA gene as isolated from the lambdaB31 clone lacked a 3' terminator region due to the cloning strategy used (Figure 4). Accordingly, a 260bp nopaline synthase (nos) BamHI-EcoRI fragment which contains an efficient plant terminator sequence was excised from the clone pNOP-NEO and ligated into BamHI-EcoRI cut pUC18 and cloned in E. coli DH5α. pNOP-NEO is a clone containing - the NOS promoter linked to the Neomycin Phosphotransferase (NPT) gene which encodes kanamycin resistance, linked to the NOS terminator as published by Bevan, Nucleic Acids Research 12, 8711-8721 (1984).

The extA gene, excised from pRlambdaS4 on a 4.75kb HindIII-SalI fragment, was then ligated into the NOS terminator pUC18 clone, also restricted with HindIII-SalI, and cloned in E. coli DH5α. The final extA - NOS terminator construct is shown in Figure 5. The whole extA - NOS terminator construct was then excised on an intact 5.01kb HindIII-EcoRI fragment and ligated into the binary vector pBIN19 (Bevan, 1984) restricted with HindIII-EcoRI, and cloned into E. coli MC1022. The final hybrid gene construct designated pBIN19:extA is shown in Figure 6 and contains additionally the endogenous kanamycin resistance gene (NPT) located between the T-DNA borders.

This construct termed pBIN19:extA was triparentally mated into A. tumefaciens LBA4404 and used to transform leaf discs of Nicotiana tabacum (SR1). Prior to tobacco being used as a test transgenic system, Southern blotting experiments were performed on tobacco DNA to show that hybridisation of extensin coding region to tobacco DNA did not occur. Even at low stringency no hybridisation of the extensin coding region to tobacco DNA could be seen. In detail:

Bacterial strains and conjugations

The vector plasmid pBIN19 in Escherichia coli strain MC1022 (Bevan, Nucl. Acids Res. 12, 8711-8721, 1984), the mobilising E. coli strain HB101/pRK2013 (Ditta et al, PNAS USA 77, 7347-7351, 1980) and the host Agrobacterium tumefaciens strain LBA4404/pAL4404 (Hoekema et al, Nature 303, 179-180, 1983) were used. For bacterial conjugation antibiotics were used at the following concentrations: kanamycin acid sulphate, $50\mu g.ml^{-1}$ (kan); rifampicin, $100\mu g.ml^{-1}$ (rif); streptomycin sulphate, $500\ \mu g.ml^{-1}$ (strep). The recombinant vector BIN19:ext A was mobilized in a triparental mating by mixing $200\mu l$ each of overnight cultures of E. coli MC1022/BIN19 ($kan^R$, $strep^S$, $rif^S$), E. coli HB101/pRK2013 ($kan^R$, $strep^S$, $rif^S$) and A. tumefaciens LBA4404 ($kan^S$, $strep^R$, $rif^R$), and incubating at 27°C on a LB plate without antibiotic selection for 15h. The cell mixture was then diluted with 10 mM $MgSO_4$; plated out on minimal medium containing kanamycin and incubated at 27°C for 4 days. The A. tumefaciens LBA4404 colonies were checked for the correct antibiotics resistance markers ($kan^R$, $strep^R$, $rif^R$), and further analysed by colony hybridisation and Southern analysis to confirm that the npt and ext A genes were present in an intact, unrearranged form in the BIN19:ext A plasmid.

Tobacco transformation and regeneration

Agrobacterium tumefaciens LBA4404/pBIN19:ext A was cultured in LB medium containing $20\mu g.ml^{-1}$ kanamycin at 27°C, the bacteria were pelleted, washed three times in 2 mM $MgSO_4$, and resuspended in MS salts (Flow Labs., Rickmansworth, Herts., UK), $10mg.ml^{-1}$ sucrose (pH 5.8) at a density of approx $10^9$ $cells.ml^{-1}$.

Expanded leaves from well-watered N. tabacum (SR1) plants were surface-sterilized with 70% (v/v) ethanol (30 s) followed by 5% (w/v) $Ca(OCl)_2$ (15 min). After washing in sterile water, each leaf was cut into squares (approx. 8 x 8

$mm^2$) avoiding major veins and then placed in the bacterial suspension, which was intermittently agitated to wet the cut leaf-edges. After 10-15 min. incubation (22-25°C) the leaf pieces were placed (adaxial surface upwards on agar plates of shoot-induction medium, comprising MS salts (including vitamins), 10 $mg.ml^{-1}$ sucrose, 2 $\mu g.ml^{-1}$ $N^6$ furfurylaminopurine (kinetin), 0.2 $\mu g/ml^{-1}$ 1-naphthylacetic acid (pH 5.8) and 8 $mg.ml^{-1}$ bacto-agar, and cultured at 25°C, 18-h photoperiod (120$\mu mol.m^{-2}.s^{-1}$ PAR). After 2 days the leaf pieces were transferred to liquid shoot-induction medium containing 1 $mg.ml^{-1}$ carbenicillin (disodium salt) and gently agitated (60 rpm shaker) overnight. The leaf pieces were surface-dried on sterile absorbant paper and placed on agar plates of shoot-induction medium containing 500 $\mu g.ml^{-1}$ carbenicillin, 200 $\mu g.ml^{-1}$ kanamycin (acid sulphate) and cultured under the conditions described above.

Shootlets which developed from the cut edges of the leaf explants (four to six weeks from inoculation) were excised and transferred to agar plates of half-strength MS salts, 5 $mg.ml^{-1}$ sucrose, 250 $\mu g.ml^{-1}$ carbenicillin, 200 $\mu g.ml^{-1}$ kanamycin (pH 5.8). The shootlets which continued to expand were transferred to 60-ml sterile vessels (Sterilin, Feltham, Middlesex, UK) containing root-induction medium comprising half-strength MS salts, 100$\mu g.ml^{-1}$ carbenicillin, 100$\mu g.ml^{-1}$ kanamycin and 8 $mg.ml^{-1}$ agar. Shootlets which developed roots were transferred to 250-ml sterile glass jars containing half-strength MS salts, 50 $\mu g.ml^{-1}$ carbenicillin and 8 $mg.ml^{-1}$ agar. When the plantlets had developed an extensive root system, they were removed from culture and potted in 1:1 Levington compost:perlite (Silvaperl Products, Harrogate, UK), and grown in a controlled environment with daily watering. Tissues from the transgenic plants were harvested into liquid air for extraction of DNA, for genomic analysis to assess the integrity of the transferred gene, and RNA, for analyses of extensin gene expression. The potted plants

flowered after about five weeks, and following self-pollination, the seeds from each plant were collected at dehiscence.

Extraction of DNA and Southern Analysis

The DNA from N. tabacum leaves was extracted and purified. The DNA probes for hybridization were $^{32}P$-labelled to high specific activities (>8 x $10^{11}$ Bq.$\mu g^{-1}$) using random priming according to Feinberg and Vogelstein (Anal. Biochem. 132, 6-13, 1983). Standard techniques were used for Southern transfer and hybridization and high stringency (30 mM NaCl, 3 mM $Na_3$ citrate, 65°C, 60 min) was employed in the final post-hybridization wash. Filters were autoradiograhed for 72 h at 80°C using flashed X-ray film (Fujimex, Swindon, UK) and intensifying screens (Du Pont, Stevenage, UK).

Extraction of RNA and Northern Analysis

Total RNAs from tissues of the transgenic N. tabacum plants were isolated by the procedure of Logemann et al. (Anal. Biochem. 163, 16-20, 1987). To estimate the size of RNAs specifically hybridizing to the rape extensin probe, 10$\mu$g of each sample RNA was glyoxalated, run on agarose gels, and transferred to nitrocellulose. Northern blots were washed to high stringency (15 mM NaCl, 1.5 mM $Na_3$ citrate, 0.1% sodium dodecyl sulphate (SDS), 25 min, 50°C), and the filters autoradiographed for 2 weeks as described above.

Fifteen transgenic tobacco plants were regenerated and grown from the initial transformation experiment. Samples of leaf and root were collected for analyses by Southern and Northern blotting. The majority of the transgenic plants regenerated contained intact, unrearranged copies of the introduced hybrid extensin gene. Estimates of the copy number of the introduced gene in the various independent transformants varied from one copy to five copies. Only transgenic tobacco plants containing intact, unrearranged copies of the introduced gene were used for

further analysis of extensin expression.

Because of the lack of availability of specific rape extensin antibodies, Northern hybridisations were performed on total RNA extracted from tobacco leaf and root tissues, using the extensin 1.0 kb coding sequence from the genomic clone lambdaB31 as a probe. No hybridisation to leaf RNA from either control or transformed tobacco plants was seen. Hybridisation of the extensin probe to transgenic root RNA was seen in all the transformants tested, although the level of hybridisation <u>between</u> individual transformants varied (position effect).

Seeds were collected from the initial batch of transformed tobacco, and were sown in compost. Plants were grown from this seed.

<u>EXAMPLE 3: Expression of Glucuronidase Enzyme under Control of the Rape Promoter</u>

In order to see the expression of the rape gene <u>extA</u> in rape, the rape promoter from lambdaB31 was fused in a translational fusion to the coding sequence of the glucuronidase (GUS) gene in a binary vector variant of BIN19. The clone containing the construct was mated with an <u>Agrobacterium</u> strain (LBA 9402) containing an oncogenic Ri plasmid, pRi 1885 (Constantino <u>et al</u>, Plasmid <u>5</u>, 170-182, 1981). Inoculation of this strain into rape seedlings produces transgenic hairy roots, which are then easily assayable for GUS activity. Whole plants can be regenerated from excised transgenic hairy roots.

In detail, the rape extensin promoter was excised from pRlambdaS4 as a 1.0kb HaeIII-HaeIII fragment. This fragment was blunt-end ligated into a modified pBIN19 binary vector, pBI101 containing a promoterless glucuronidase (GUS) gene, restricted with SmaI at the start of the GUS coding sequence (see Jefferson, Plant Molecular Biology Reporter <u>5</u>, 387-405, 1987) (Figure 8). The rape promoter expression from this translational fusion will give a glucuronidase

enzyme with either 7 or 11 extra amino acids at the N-terminus, depending on which rape ATG is used as the translation initiation codon.

The extA-GUS hybrid gene in pBI101 (pBI101:extA-GUS) was cloned in E. coli MC1022 and then transferred into Agrobacterium tumefaciens or Agrobacterium rhizogenes by tri-parental matings with the appropriate Agrobacterium strains. Preliminary results to test for expression from this construct, using the X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) histochemical assay of Jefferson (Plant Molecular Biology Reporter 5, 387-405, 1987) in rape 'hairy roots' transformed with Agrobacterium rhizogenes containing pBI101:extA-GUS, have clearly shown that the promoter is active in rape root tissue.

Rape Tissue culture and transformation protocols:

Transformation experiments using A. rhizogenes

Axenically growing seedlings of B. napus cv. Bienvenue (winter rape), Brutor and Rapid Cycling were inoculated at their cotyledonary nodes with an A. rhizogenes LBA9402 strain carrying both the Ri plasmid pRi 1855 (Constantino et al, 1981) and the kanamycin resistance fusion construct of Figure 8 (pBI101:extA-GUS) as separate independent replicons. Hairy roots arising from the site of inoculation can be excised and grown in a culture medium (1 x MS salts and vitamins, 4% sucrose, 2 g/l $CaCl_2.H_2O$, 0.1 mg/l NAA, 2.5 mg/l BAP) 0.1 mg/l thiamine, 200 mg/l cephotaxime, 50 mg/l kanamycin, 8 g/l Bacto Difco agar, pH 5.8, to proliferate the hairy roots.

Regeneration can be achieved as follows: 0.5 cm sections of the terminal 2 - 3 cm of hairy roots are excised, treated with 2,4 - D at a concentration of 3 mg/l, and plated on shooting medium containing kanamycin at 50 mg/l to select for tissues which contained the T-DNA kanamycin gene of the fusion construct of Figure 8. Shoots arising from these hairy roots are excised and grown on medium F of Pelletier et al (Mol. Gen. Genet. 191, 244-250

(1983)) for 2-3 weeks. They are then transferred to rooting medium G, and when a sufficient root system has developed they are transferred to soil and grown to maturity in a containment growth room.

Transgenic winter rape plants are vernalised to induce flowering by growing them at 4°C with a 8h daylight period. Flowers are emasculated before anthesis and outcrossed with wild type rape pollen. Seeds obtained after pod set are collected, sown in soil and the progeny plants grown to maturity. The progeny plants are initially scored visually with regard to the degree of the hairy root syndrome being exhibited. Kanamycin resistance in the leaves of the progeny is assayed by transferring leaf discs to callus inducing media (see below) containing kanamycin at either 50 or 150 mg/l. Both the Ri and the kanamycin resistance traits can be further confirmed by Southern hybridisation analysis.

The media used in the Examples are:

Media

1. For seedling growth

| | | |
|---|---|---|
| MS salts | 2.355 g/l | |
| Sucrose | 20 g/l | |
| Difco Bacto agar | 15 g/l | pH 5.8 |

2. For Agrobacterium rhizogenes liquid culture - YMB

| | | |
|---|---|---|
| Mannitol | 10 g/l | |
| Yeast extract | 0.4 g/l | |
| $KH_2PO_4$ | 0.5 g/l | |
| $MgSO_4.2H_2O$ | 0.2 g/l | |
| NaCl | 0.1 g/l | |
| Kanamycin | 50 mg/l | pH 7.0 |

3. For hairy root culture (Ooms' medium: Ooms et al (1985) Theor. Appl. Genet. 71, 325-329)

| | |
|---|---|
| MS salts | 4.71 g/l |
| $CaCl_2.2H_2O$ | 2 g/l |
| Sucrose | 40 g/l |
| NAA | 0.18 mg/l |
| BAP | 2.5 mg/l |
| Thiamine HCl | 0.1 mg/l |
| Difco Bacto agar | 8 g/l |
| Kanamycin | 25 or 50 mg/l |
| Cephotaxime | 200 mg/l pH 5.8 |

4. For shoot induction, multiplication and rooting

D solution, RCC medium, F and G media are as described by Pelletier et al. (1983) and Guerche et al. (Mol. Gen. Genet. 206, 382-386, 1987). In addition, a level of Cephotaxime of 200 mg/l is maintained in all these media at all times.

5. Callusing medium for transformed N. tabacum and rape leaf discs

MS3SC 100 kan.

| | |
|---|---|
| MS salts | 4.7 g/l |
| Sucrose | 30 g/l |
| NAA | 2 mg/l |
| Kinetin | 0.2 mg/l |
| Kanamycin | 100 mg/l |
| Agar | 8 g/l |

CLAIMS

1. A promoter which is capable of directing protein expression in roots in plants and which has:

(a) the sequence from nucleotide -1616 to nucleotide -1 shown in Figure 1 or a part of the said sequence or

(b) a said sequence (a) modified by one or more nucleotide substitutions, insertions and/or deletions and/or by an extension at either or each end.

2. A DNA fragment comprising a promoter as claimed in claim 1 operably linked to a gene encoding a protein.

3. A vector which comprises a gene, encoding a protein, under the control of a promoter as claimed in claim 1, such that the gene is capable of being expressed in a plant cell transformed with the vector.

4. A vector according to claim 3, wherein the promoter is fused directly to the 5'-end of the said gene.

5. A vector according to claim 3 or 4, which further contains a region which enables the gene and the promoter to be transferred to and stably integrated in a plant cell genome.

6. A vector according to any one of claims 3 to 5, which is a plasmid.

7. A plant cell which has been transformed with a vector as claimed in any one of claims 3 to 6.

8. A plant cell which harbours a promoter as claimed in claim 1 operably linked to a gene encoding a protein.

9. A transgenic plant which has been regenerated from plant cells as claimed in claim 7 or 8.

10. A transgenic plant which harbours in its cells a promoter as claimed in claim 1 operably linked to a gene encoding a protein.

11. Seed obtained from a transgenic plant as claimed in claim 9 or 10.

12. Plants grown from seed as claimed in claim 11.

13. A method of producing a transgenic plant capable of producing a desired protein in the roots of the plant, which method comprises:

(i) transforming a plant cell with a vector as claimed in any one of claims 3 to 6, the protein encoded by the gene under the control of the said promoter being the desired protein; and

(ii) regenerating plants from the transformed cells.

# Fig.1 Cont.

```
                                                                                  ..........
extA ATACCATCAGGGATTAAAAAAGTGAAATTGAAAAGCTATATAAAGGTGATTAGAGGGAGAGAGTAAGAGCATCAAACACAAAGAAAAACACATAACAATATGGGGTCACCAATGGCCTCT  50
     ...................................<TATA BOX>......................<T^S>....................................<M  A  S  -21

extA TTGGCAGCAACTTTGCTTGTCTTAGCACTTTCTCTTGGTTTTGTATCTGAAACCACCGCAAATTACTACTACTCTTCTCCTCCTCCACCGGTCAAACACTACACTCCTCCGGTGTACAAG 170
      L  A  A  T  L  L  V  L  A  L  S  L  G  F  V  S  E  T  T  A :N  Y  Y  Y  S  S  P  P  P  P  V  K  H  Y  T  P  P  V  Y  K   20

extA TCTCCTCCACCACCGGTAAAGCACTACTCTCCTCCGGTTTACAAATCCCCACCTCCACCAAAGAAGGACTACGAGTACAAATCACCTCCACCACCGGTCAAGCATTACTCTCCTCCTCCA 290
      S  P  P  P  P  V  K  H  Y  S  P  P  V  Y  K  S  P  P  P  P  K  K  D  Y  E  Y  K  S  P  P  P  P  V  K  H  Y  S  P  P  P   60

extA GTTTACAAGTCTCCACCTCCTCCCAAGAAACATTACGAGTACAAATCACCTCCTCCACCGGTTTACAAATCTCCTCCTCCTCCGGTTTACCACTCACCTCCACCACCTAAAAAACACTAC 410
      V  Y  K  S  P  P  P  P  K  K  H  Y  E  Y  K  S  P  P  P  P  V  Y  K  S  P  P  P  P  V  Y  H  S  P  P  P  P  K  K  H  Y  100

extA GAGTACAAATCACCTCCTCCACCGGTTTACAAGTCTCCTCCCCCTCCGGTCTACCACTCTCCTCCACCACCTAAGAAACACTACGAGTACAAATCTCCTCCTCCACCGGTTTACAAGTCT 530
      E  Y  K  S  P  P  P  P  V  Y  K  S  P  P  P  P  V  Y  H  S  P  P  P  P  K  K  H  Y  E  Y  K  S  P  P  P  P  V  Y  K  S  140

extA CCTCCTCCTCCAGTCTACCACTCTCCTCCACCACCTAAGAAACACTACGAGTACAAATCACCTCCTCCACCGGTTTACAAGTCTCCTCCTCCTCCAGTCTACCACTCTCCCCCACCACCT 650
      P  P  P  P  V  Y  H  S  P  P  P  P  K  K  H  Y  E  Y  K  S  P  P  P  P  V  Y  K  S  P  P  P  P  V  Y  H  S  P  P  P  P  180

extA AAGAAACACTACGAGTACAAATCTCCTCCTCCACCGGTTTACAAGTCTCCTCCTCCTCCGGTTTACCACTCTCCTCCACCACCTAAGAAGCATTACGAGTACAAATCTCCTCCTCCTCCG 770
      K  K  H  Y  E  Y  K  S  P  P  P  P  V  Y  K  S  P  P  P  P  V  Y  H  S  P  P  P  P  K  K  H  Y  E  Y  K  S  P  P  P  P  220

extA GTTTACCAGTCTCCTCCCCCTCCGGTTTACCACTCTCCCCCACCACCAAAGAAACACTACGAATACAAATCGCCGCCACCTCCGGTCTATTCTCCTCCTCCACCGGTTCACTACTCACCT 890
      V  Y  Q  S  P  P  P  P  V  Y  H  S  P  P  P  P  K  K  H  Y  E  Y  K  S  P  P  P  P  V  Y  S  P  P  P  P  V  H  Y  S  P  260

extA CCTCACCACCCCTACCTTTACAAATCTCCACCTCCTCCATACCACTACTAGATAAACTGATCCGTTGACCTGCAG>[end of genomic clone]
      P  H  H  P  Y  L  Y  K  S  P  P  P  P  Y  H  Y  *>.......................
```

# Fig. 1.

```
extA ..............AACTAAATTCCAAGGAGAAAATTGGCTGTAAAATGAATTATTTCCACAGTTTGTATTTATTTATAAATGCATGGACATTATATATAAATTAAGACACAGTGTATAT -1511
extA ATTTTATATCTTTCTCATTGAGTTGTTTACATCGTTTCATATCTCTTTCTCTAATGCAGTTAAGAAATCTAGCAGAAATTTTTTTTTAGTCTAGCAAAACATTATAACATATTTTGTCT -1391
extA TAATCTCAAACGTATTTGAACTCGTTTAATGTTGATTGAATAATAATTGAGAGTAAGAGAATACAAACTAATCTGAGTAATAATGACAAGAAACGTACGTGTTATAAAACGTGTGAAGCA -1271
extA AAATCTAACCCTAAAACAAACAGAAAATAATTAAGATTGACTGTCACCGGCTCAAGAGAACGACGGACCAAACTTCTCCTTCAATGATCCTTCTTTATTCAACTCGGCCGCCTAACTTTA -1151
extA ATCTTCAAGACTTTAACGCGGCTTCTAGTTTTCTATATCCAATAAATCGGTAAACAAAACAAAATCTTTGAATAATGTAATTATTACCCAGGACCTCGTAGTCCAGTGGTACTACCTTCT -1031
extA CTTTGAGGAGGGAAGGTCGGTTGTTCGACTCGCGGGGAGGGGGAGCGTGCCCAGGACCCTAACCGGTACAGGCACACAGCTGGCGCCGGGCCTAGGTGGTAGACTGGTCGAAGGCTGGTC -911
extA AACACCTGGTTAAACAAAAAAAAAAAAAAAATAATAATAATAATGTAATTATTAATTAAATAAAATTAAAAAAATTAAAATCTTGATTTATAAATATAAGTTTAAGAAGATGTCGTAACTG -791
extA ATAAAAAAAAATCGTGATAGTGCGTAAATATTCTATATACATAATGTTGGGCAAATGATGAAATCACAACATATATATATATATATAGCAGTTTTTAATTTATTAATAAAATATCAATTTTTA -671
extA CGAAAAATACATAAAAGTTTATTCGCATATGATAGATGTCCGCGAGATTATGCCAAATAATAGAAAATTAAGTTATTAATTAAATAAAAAATACTGCAATATGTACAGTATTAGTCTTATT -551
extA TCTGTTAGTAGTGTGTTGATCAATATATCTAATATTGATGTAAAACATATACAAACATTCCAACTAAAATATAGTGGATTTTGCATATGAATATCTATCATATGACATGGCTTCAATACT -431
extA CTAAGTTATACTGTTTTGATTTAAGTGTCATAATAGTTTGTGTGACTATATATTATTGATGCGTTAGTATTCAGATTCAAACTCGTATATCCATCTTTTTGAAAGATAACATTTTAATAA -311
extA ATAAAAATAAAATACTGTAAGGTATTTTCATTTCCTGGTAAATAAACTCACAAATACAGTAGTTACATTTACCACTCAGAACGTGTTATTATTTTCATACTTTCCATCTTTTTTTTTCTTA -191
extA CCAATTGCAAGCATAATGTCGGTCGTTTTTTGTGTGTGGCTCAATAATGAAGTTTGAGAGAGTAACCTCATTTCTTTTACATAGTCAATTATTGTTGTTTATTACATATGACAAAAATGC -71
```

Fig. 2.

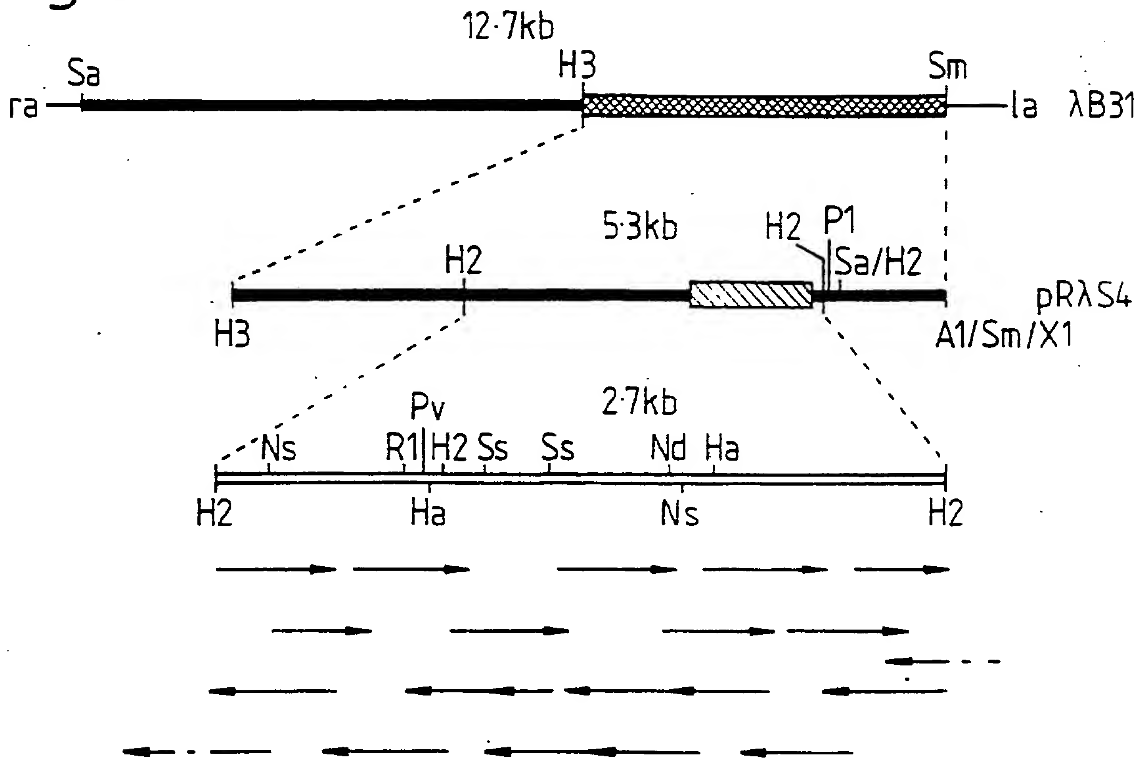



Fig. 7.


Fig. 8.

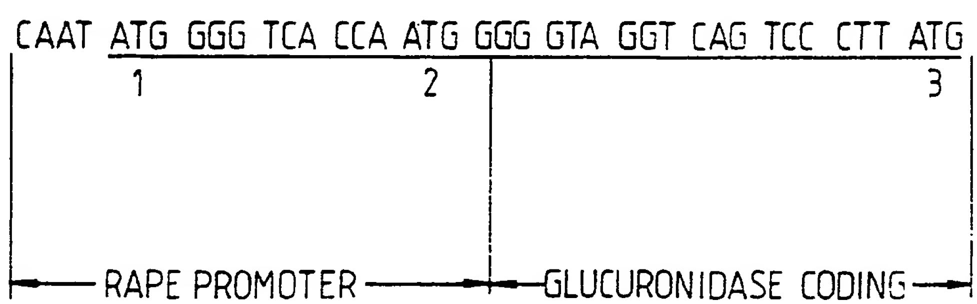
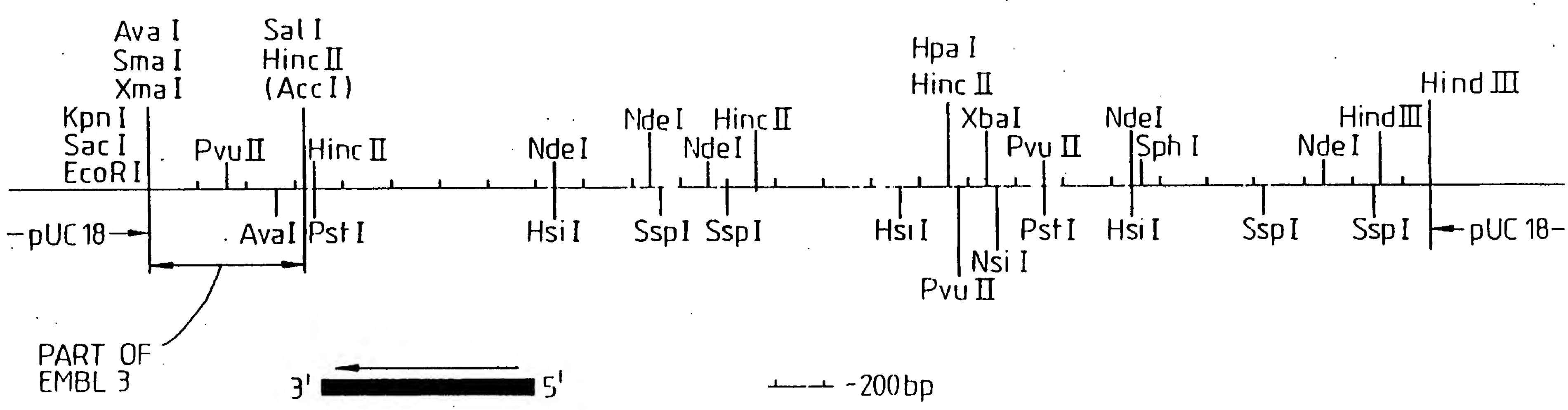


*Fig. 3.*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Sac I
EcoR I
Ava I
Sma I
Xma I
Pvu II
Sal I
Hinc II
(Acc I)
Ava I
Hinc II
Pst I
-pUC 18-
PART OF
EMBL 3
Nde I
Hsi I
Nde I
Ssp I
Nde I
Ssp I
Hinc II
Hsi I
Hpa I
Hinc II
Pvu II
Xba I
Nsi I
Pvu II
Pst I
Nde I
Sph I
Hsi I
Ssp I
Nde I
Hind III
Ssp I
Hind III
-pUC 18-
3'
5'
~200bp

Fig. 4.

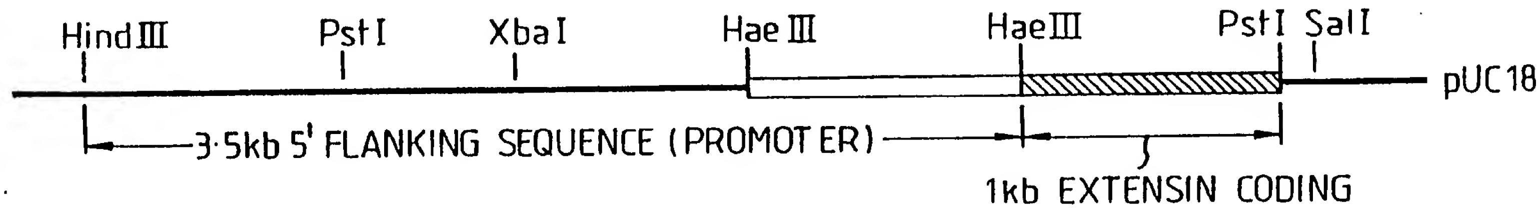



Fig. 5.

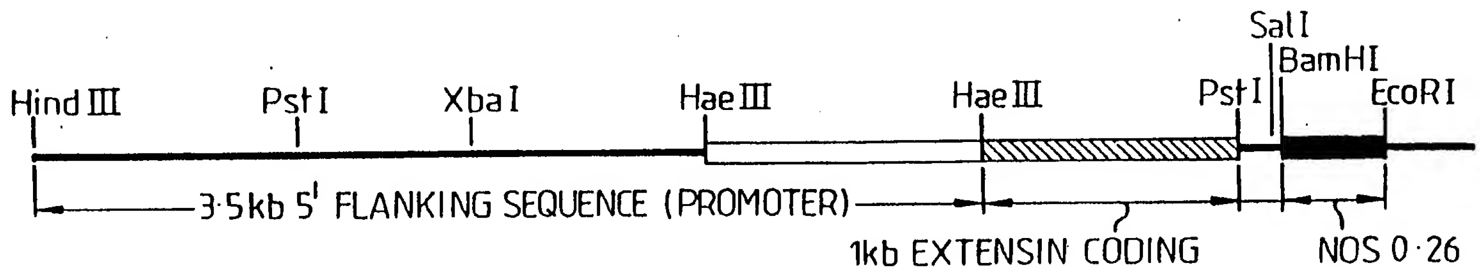
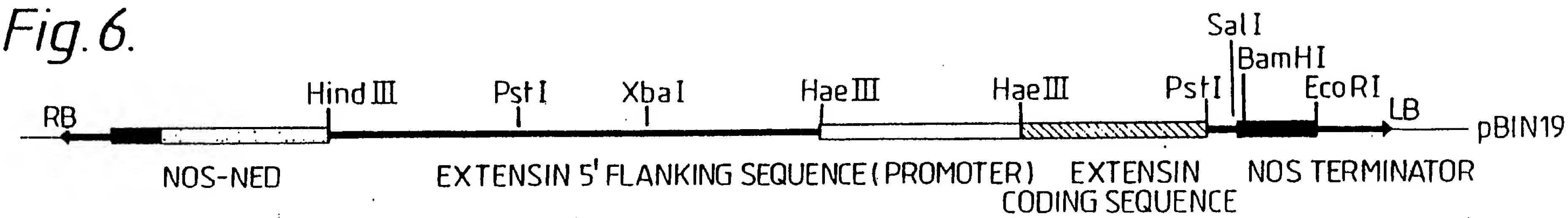



Fig. 6.



# INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 91/00416

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC$^5$: C 12 N 15/82, 15/29, 5/10, A 01 H 5/00, A 01 H 5/10

**II. FIELDS SEARCHED**

Minimum Documentation Searched [7]

| Classification System | Classification Symbols |
|---|---|
| IPC$^5$ | C 12 N, A 01 H |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

**III. DOCUMENTS CONSIDERED TO BE RELEVANT** [9]

| Category * | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| A | Chemical Abstracts, volume 112, 1990, (Columbus, Ohio, US), Keller, Beat et al.: "Specific expression of a novel cell wall hydroxyproline-rich glycoprotein gene in lateral root initiation", see page 209, abstract no. 113287y & Genes Dev. 1989, 3(10), 1639-46 --- | 1-11 |
| A | Chemical Abstracts, volume 110, 1989, (Columbus, Ohio, US), A.L. Gartel et al.: "Cloning of the β-glucuronidase gene under the control of the carrot extensin gene promoter", see page 225, abstract no. 207159u & Dokl. Vses. Akad S-kh. Nauk im. V.I. Lenina 1989, (3), 7-9 --- | 1-11 |

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

**IV. CERTIFICATION**

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 14th June 1991 | 22 JUL 1991 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | MISS T. TAZELAAR |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, [11] with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
|---|---|---|
| P,X | Mol. Gen. Genet, volume 223, 1990, MGG, Springer Verlag, Marta Evans et al.: "The extensin gene family in oilseed rape (Brassica napus L.): Characterisation of sequences of representative members of the family", pages 273-287 see the whole document | 1-11 |
| A | The Plant Cell, volume 1, January 1989, American Society of Plant Physiologists R.A. Dietrich et al. "Spatial patterns of gene expression in Brassica napus seedlings: Identification of a cortex-specific gene and localization of mRNAs encoding isocitrate lyase and a polypeptide homologous to proteinases", pages 73-80 see page 75, right-hand column | 1-11 |

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